

## **ISOLATION OF THE LAMININ $\gamma$ 2 GENE IN HORSES AND ITS USE IN DIAGNOSING JUNCTIONAL EPIDERMOLYSIS BULLOSA**

### **Field of the Invention**

[0001] The present invention relates to the isolation of the gene encoding the  $\gamma$ 2 subunit of equine laminin-5 and its use in diagnosing junctional epidermolysis bullosa (JEB) in horses.

### **Background of the Invention**

[0002] Epidermolysis bullosa (EB) is a group of hereditary and acquired diseases of the skin and mucous membranes that share the common feature of the formation of blisters and erosions in response to minor trauma (Fine et al., 2000).

[0003] In humans, the clinical forms of hereditary EB are divided into three main categories, each typified by the level of skin separation within the dermal-epidermal basement membrane zone and by the proteins involved. EB simplex (EBS) is characterized by separation occurring in the basal keratinocytes due to mutations in the keratin 5 and 14 genes or in the hemidesmosomal component plectin (Fuchs, 1992). In dystrophic EB (DEB), the skin separates at the lower layer of the basement membrane zone, the lamina densa, as a result of defects in anchoring fibrils (Uitto and Christiano, 1993). In junctional EB (JEB), blister formation takes place within the lamina lucida of the dermal-epidermal basement membrane and several mutations have been described in the three genes (LAMA3, LAMB3, and LAMC2) that encode the anchoring filament protein, laminin 5, and the two transmembrane components of the hemidesmosome (HD), collagen XVII and integrin  $\alpha$ 6 $\beta$ 4. Among the JEB variants, the non-Herlitz or mild forms of JEB (non-H JEB) are characterized by chronic and localized blistering with non-shortening of the patient's life span. Hemidesmosomes are present but usually reduced in number. Herlitz JEB (H-JEB) represents the most severe and the most frequent form of JEB (greater than 50% of cases). H-JEB is characterized by generalized blistering with erosions of the skin and mucous membranes, and is lethal in early childhood. Ultrastructural and immunohistochemical observations demonstrate abnormalities in hemidesmosome anchoring filaments complexes. Immunostaining of the skin of patients affected by H-JEB reveals absence of laminin-5.

[0004] Laminin 5 is synthesized within the basal epithelial cells as a heterotrimeric molecule composed of an  $\alpha$ 3 (200 kDa), a  $\beta$ 3 (140 kDa) and a  $\gamma$ 2 (155 kDa) chain that associate to form a triple-stranded  $\alpha$ -helical coiled-coil rod domain (Engel, 1991). A large number of distinct mutations (greater than 100) have been identified in the three genes encoding the polypeptide subunits of laminin 5 (Pulkkinen and Uitto, 1999).

- [0005] Animal models for mechanobullous disorders have been described in the literature, including transgenic mouse models and xenograft models but naturally-occurring, well-characterized animal models are rare.
- [0006] Clinical reports of sporadic cases of hereditary EB have been described in a range of animals including sheep (Bruckner-Tuderman), cattle, cats, dogs and horses, but electron microscopy examination was often absent, the breeding history of animals incomplete and the inheritance mode of the disease unclear. Clinical features observed in humans often differ in animals and this is probably due to differences in the skin characteristics among species.
- [0007] In order to understand the molecular basis of this disease in horses, with a view to minimizing its occurrence, it would be desirable to clone the relevant genes in an attempt to determine the cause of one or more forms of EB in horses.

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### Summary of the Invention

- [0008] In one aspect of the present invention, an isolated polynucleotide encoding the  $\gamma 2$  subunit of laminin-5 in horses is provided.
- [0009] In another aspect of the present invention, a method for diagnosing JEB in horses is provided comprising the steps of:
- 1) obtaining a biological sample from a horse;
  - 2) isolating nucleic acid from the sample and amplifying laminin  $\gamma 2$ -encoding polynucleotide using appropriate primers; and
  - 3) sequencing the laminin  $\gamma 2$ -encoding polynucleotide, wherein an inserted cytosine at position 1368 is indicative of JEB.
- [0010] In another aspect of the present invention, there is provided a method of diagnosing JEB in horses comprising:
- 1) obtaining a biological sample from a horse;
  - 2) isolating protein from the sample; and
  - 3) screening the sample for laminin- $\gamma 2$  peptide, wherein an absence of laminin- $\gamma 2$  in the sample is indicative of JEB.
- [0011] In a further aspect of the present invention, a kit for diagnosing JEB in horses is provided. The kit includes the primers, 5'-TGTTACTCAGGGATGAGAA-3' (SEQ ID No: 29) and (antisense) 5'-CTGGGGGCAGTTATTGCAC-3' (SEQ ID No: 30) for use in amplifying laminin  $\gamma 2$  nucleic acid.
- [0012] These and other aspects of the present invention are described by reference to the following figures in which:

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### **Brief Description of the Figures**

- [0013] Figure 1 illustrates the clinical features of JEB in Belgian horses;
- [0014] Figure 2 illustrates the altered expression of laminin  $\gamma 2$  in horse JEB using an immunofluorescence analysis of frozen sections of tongue epithelia samples obtained from an affected foal (a, c, e, g) and a healthy unrelated control (b, d, f, h);
- [0015] Figure 3 illustrates the nucleotide (SEQ ID No: 1) and deduced amino acid (SEQ ID No:2) sequence of the horse laminin  $\gamma 2$  chain;
- [0016] Figure 4. Direct comparison of the primary structure of the horse (upper line), human (middle line) and mouse (lower line) laminin  $\gamma 2$  chain;
- [0017] Figure 5 provides a chromatographic comparison of the genomic sequences in which the homozygous insert mutation at position 1368 (1368insC) of the DNA sequence (panel A), the heterozygous situation for the mutation 1368insC in a carrier horse (panel B) and the wild-type DNA sequence (panel C) are shown; and
- [0018] Figure 6 illustrates the inheritance of the laminin  $\gamma 2$  mutant allele in Belgian horses.

### **Detailed Description of the Invention**

- [0019] Junctional epidermolysis bullosa (JEB) in horses has been linked to the  $\gamma 2$  subunit of the laminin-5 gene. A  $\gamma 2$ -encoding polynucleotide has been cloned and sequenced in accordance with an aspect of the present invention. The mutation associated with the clinical signs of JEB in horses results in a homozygous nucleotide insertion in the laminin  $\gamma 2$ -encoding polynucleotide, a frame shift, and a premature termination codon. Specifically, a cytosine insert occurs in the genomic nucleic acid sequence of affected horses at position 1368 of the laminin  $\gamma 2$ -encoding polynucleotide.
- [0020] As used herein, the term “laminin  $\gamma 2$ ” is meant to refer to the “ $\gamma 2$ ” or “LAMC2” subunit of the anchoring filament protein, laminin-5.
- [0021] The isolated laminin  $\gamma 2$  polynucleotide comprises a 3570-bp full-length open reading frame, the sequence of which is set out in SEQ ID NO: 1 (Fig.3). The polynucleotide encodes a polypeptide consisting of 1190 amino acid residues in its mature form, as identified by three-letter code in SEQ ID NO: 2 (Fig. 3).
- [0022] Laminin  $\gamma 2$  -encoding nucleic acid can be prepared by applying selected techniques of gene isolation or gene synthesis as a first step. As described in more detail in the examples herein, laminin  $\gamma 2$  polynucleotides can be obtained by careful application of conventional gene isolation and cloning techniques such as the homologous RT(PCR) amplification technique. Gene cloning

can also be conducted by extraction of total messenger RNA from an appropriate tissue source, such as skin or hair follicles, followed by conversion of message to cDNA and formation of a cDNA library in plasmidic vectors. The cDNA library is then probed using a labelled nucleic acid fragment derived from a gene believed to be highly homologous to the cDNA of interest. Hybridizing cDNA clones are further screened and positive clones are prepared for insertion into an expression vector.

- [0023] Having herein provided the nucleotide sequence of a polynucleotide encoding laminin  $\gamma$ 2 (Fig. 3), it will be appreciated that automated techniques of gene synthesis and/or amplification can be performed to generate laminin  $\gamma$ 2-encoding DNA. In this case, because of the length of the laminin  $\gamma$ 2-encoding DNA, application of automated synthesis may require staged gene construction in which regions of the gene are synthesized individually and then ligated in correct succession via designed overlaps.
- [0024] PCR amplification may be used to directly generate all or part of the final gene. In this case, primers are synthesized which will prime the RT-(PCR) amplification of the final product, either in one piece, or in several pieces that may subsequently be ligated together via step-wise ligation of blunt ended, amplified DNA fragments, or preferentially via step-wise ligation of fragments containing naturally occurring restriction endonuclease sites.
- [0025] Both cDNA or genomic DNA are suitable as templates for PCR amplification. The former may be obtained from a number of sources including commercially available cDNA libraries, single- or double-stranded cDNA, or cDNA constructed from isolated messenger RNA from a suitable tissue sample. Genomic DNA, obtained from blood or any tissue sample, may also be used as a template for the PCR-based amplification of the gene; however, the gene sequence of such genomic DNA may contain unwanted intervening sequences.
- [0026] Once obtained, the laminin  $\gamma$ 2-encoding DNA is incorporated for expression into any suitable expression vector, and host cells are transfected therewith using conventional procedures. The particular cell type selected to serve as host for production of laminin  $\gamma$ 2 can be any of several cell types currently available in the art, including both prokaryotic and eukaryotic cell types. Chinese hamster ovary (CHO) cells for example of K1 lineage (ATCC CCL 61) including the Pro5 variant (ATCC CRL 1281); the fibroblast-like cells derived from SV40-transformed African Green monkey kidney of the CV-1 lineage (ATCC CCL 70), of the COS-1 lineage (ATCC CRL 1650) and of the COS-7 lineage (ATCC CRL 1651); murine L-cells, murine 3T3 cells (ATCC CRL 1658), murine C127 cells, human embryonic kidney cells of the 293 lineage (ATCC CRL 1573), human carcinoma cells including those of the HeLa lineage (ATCC CCL 2), and neuroblastoma

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cells of the lines IMR-32 (ATCC CCL 127), SK-N-MC (ATCC HTB 10) and SK-N-SH (ATCC HTB 11) all represent examples of suitable cell types for the production of mammalian laminin  $\gamma 2$ .

- [0027] A variety of gene expression systems have been developed and are now commercially available. Any one of these systems can be selected to drive expression of the laminin  $\gamma 2$ -encoding DNA. These systems, available typically in the form of plasmidic vectors, carry expression cassettes which include DNA expression controlling sequences, which are host-recognized and enable expression of laminin  $\gamma 2$ -encoding DNA when linked 5' thereof. Laminin  $\gamma 2$ -encoding DNA is herein referred to as being incorporated "expressibly" into the system, and incorporated "expressibly" in a cell once successful expression from a cell is achieved. These systems further incorporate DNA sequences which terminate expression when linked 3' of the coding region. Thus, for expression in the selected cell host, there is generated a recombinant DNA expression construct in which the laminin  $\gamma 2$ -encoding DNA is linked with expression controlling DNA sequences recognized by the host, and which include a region 5' of the laminin  $\gamma 2$ -encoding DNA to drive expression, and a 3' region to terminate expression.
- [0028] Included among the various recombinant DNA expression systems that can be used to achieve mammalian cell expression of the laminin  $\gamma 2$ -encoding DNA are those that exploit promoters of viruses that infect mammalian cells, such as the promoter from cytomegalovirus (CMV), the Rous sarcoma virus (RSV), simian virus (SV40), murine mammary tumor virus (MMTV) and others. Also useful to drive expression are promoters such as the LTR of retroviruses, insect cell promoters, including those isolated from *Drosophila* which are regulated by temperature, as well as mammalian gene promoters such as those regulated by heavy metals, i.e. the metallothionein gene promoter, and other steroid-inducible promoters.
- [0029] Expression systems may be selected to provide transformed cell lines that express the laminin  $\gamma 2$ -encoding DNA in a stable manner. Suitable expression vectors will typically harbour a gene coding for a product that confers on the transformants a survival advantage to enable their subsequent selection. Genes coding for such selectable markers include the *E. coli* gpt gene which confers resistance to mycophenolic acid, the neo<sup>R</sup> gene from transposon Tn5 which confers resistance to neomycin and to the neomycin analog G418, the dhfr sequence from murine cells or *E. coli* which changes the phenotype of DHFR- cells into DHFR+ cells, and the tk gene of herpes simplex virus, which makes TK- cells phenotypically TK+ cells. Other methods of selecting for transformants may of course be used, if desired, including selection by morphological parameters, or detection of surface antigen or receptor expression. The latter can be monitored using specifically labelled antibodies and a cell-sorter, e.g. fluorescent activated.

- [0030] The present invention also provides, in another of its aspects, antibody to laminin- $\gamma$ 2. To raise such antibodies, there may be used as immunogen either full-length laminin- $\gamma$ 2 or an immunogenic fragment thereof, produced in a microbial or mammalian cell host as described above or by standard peptide synthesis techniques. Regions of laminin- $\gamma$ 2 particularly suitable for use as immunogenic fragments include regions which are determined to have a high degree of antigenicity based on a number of factors, as would be appreciated by those of skill in the art, including for example, amino acid residue content, hydrophobicity/hydrophilicity and secondary structure. Specific examples of immunogenic fragments of laminin- $\gamma$ 2 suitable for generating antibodies include, but are not limited to, the region spanning residues 1-200, the region spanning residues 380-610, and the region spanning residues 800-1190.
- [0031] The raising of antibodies to mammalian laminin- $\gamma$ 2 or to desired immunogenic fragments can be achieved, for polyclonal antibody production, using immunization protocols of conventional design, and any of a variety of mammalian hosts, such as sheep, goats and rabbits. Alternatively, for monoclonal antibody production, immunocytes such as splenocytes can be recovered from the immunized animal and fused, using hybridoma technology, to myeloma cells. The fusion cell products, i.e. hybridoma cells, are then screened by culturing in a selection medium, and cells producing the desired antibody are recovered for continuous growth, and antibody recovery. Recovered antibody can then be coupled covalently to a reporter molecule, i.e. a detectable label, such as a radiolabel, enzyme label, luminescent label or the like, using linker technology established for this purpose, to form a specific probe for laminin- $\gamma$ 2.
- [0032] According to another aspect of the present invention, DNA or RNA encoding equine laminin  $\gamma$ 2, and selected regions thereof, may also be used in detectably labelled form, e.g. radiolabelled or fluorescently labelled form, as hybridization probes to identify sequence-related genes existing in other mammalian genomes (or cDNA libraries). This can be done using the intact coding region, due to a high level of conservation expected between related genes, or by using a highly conserved fragment thereof, having radiolabeled nucleotides, for example,  $^{32}\text{P}$  nucleotides, incorporated therein.
- [0033] In a further aspect of the present invention, there is provided a method for diagnosing JEB in a horse. Although this method can be used to diagnose JEB in foals post-natally, affected animals can usually be visually diagnosed at or within days of birth. Accordingly, the method is most usefully applied prenatally, to determine whether or not an unborn foal is affected. A positive prenatal diagnosis provides a breeder with the opportunity to terminate a pregnancy that will result in the birth of a fatally affected foal. The method involves obtaining a biological sample from the horse to be diagnosed, or fetus in the case of prenatal diagnosis. For post-natal diagnosis the

biological sample may be any nucleic acid or protein-containing sample, depending on the method to be used as is outlined in more detail below. Preferably, the biological sample is non-invasively obtained, including samples such as skin or hair follicles. Prenatal diagnosis is necessarily invasive. Examples of suitable biological samples for use in prenatal testing include fetal blood, skin or chorionic villosities.

- [0034] In one embodiment, upon obtaining the biological sample, nucleic acid is isolated therefrom using techniques well-known to those of skill in the art. The isolated nucleic acid is then screened using specifically designed labelled probes to detect whether or not the sample contains the disease-indicating allele which is defined herein as a laminin  $\gamma 2$ -encoding polynucleotide having a cytosine inserted at position 1368, thereby resulting in a termination codon at position 476. Using methods well-established in the art, hybridizing probes are detected and a positive diagnosis is made for an affected homozygous animal. A positive diagnosis will also result for a healthy heterozygous carrier due to the presence of the morbid allele. Accordingly, positive samples must be further analysed to determine if the animal is homozygous or heterozygous with respect to the mutated laminin  $\gamma 2$  gene. As is described in more detail in the specific examples that follow, direct nucleotide sequencing followed by chromatographic analysis of the genomic DNA is one way in which the homozygous and heterozygous existence of the mutated laminin  $\gamma 2$  alleles can be differentiated to confirm a JEB diagnosis in the homozygous case. As will be understood by those of skill in the art, the chromatographic analysis may be conducted in the absence of nucleotide sequencing in order to diagnose JEB or heterozygous carriers of JEB and to identify wild type animals which can then be selected for reproduction. Moreover, the present diagnostic method can advantageously be used, particularly post-natally to identify carriers of the mutation associated with JEB, in order that such carriers can be removed from the breeding population and thereby minimize the occurrence of JEB in offspring.
- [0035] In another embodiment, a method of diagnosing JEB in horses is provided in which the protein component of the biological sample is isolated using conventional methods. The protein component is then analysed to determine whether or not it contains the laminin  $\gamma 2$  peptide. The absence of such a peptide indicates the presence of the mutated laminin  $\gamma 2$  gene (comprising a cytosine insert at nucleic acid position 1368 resulting in a premature termination codon at amino acid position 476 in the peptide) and represents a positive JEB diagnosis.
- [0036] Embodiments of the present invention are described by reference to the following specific examples which are not to be construed as limiting.

**Example 1 - Clinicopathological observations and immunochemistry associated with JEB in horses**

**Clinicopathological Observations**

- [0037] Belgian horses suffering from extensive skin blistering with severe oral cavity involvement were used for observation. Foal n° 1 was the fourth foal of a mare that had previously had an affected offspring. One day after birth, multiple ulcerations of the skin were present over the pressure points on the legs and head as shown in Fig. 1A. Ulcers were also observed on the tongue, and the mucous membrane of rostral maxillae as shown in Fig. 1D. Foal n° 2 presented multiple mucosal and epithelial erosions at birth. Multifocal, irregular areas of erosions were noted over the pressure points of the extremities, around the coronary band of all four hooves (Fig. 1B), and on the oral and conjunctival mucous membranes. An abnormal roughened enamel was present over the teeth and there was evidence of bleeding from oral erosions (Fig. 1C). In foal n° 3, multiple skin defects were noticed shortly after birth. Eight days after birth, large, extensive and confluent ulcerated areas were present over the pressure points of the body. The right front hoof had detached and the underlying lamina was exposed. The buccal mucous membranes were hyperemic. Due to the grave prognosis, the foal was euthanized and submitted for necropsy. One day after birth, foal n° 4 presented ulcerated skin lesions over the limbs and at the base of the hooves. Extensive ulcerations of the oral cavity were noted over the gingival mucosa and the soft palate. Teeth were visible and dysplastic, with white serrated edges.
- [0038] The clinical observations were consistent with the characteristics of epidermolysis bullosa.

**Immunochemistry**

- [0039] Indirect immunofluorescence analysis was performed on 5 µm sections of the frozen tongue samples using antibodies raised against the human basement membrane components and cross-reacting with the horse counterparts. Polyclonal antibody (pAb) SE85 is specific to the laminin α3 chain [Vidal, 1995]; pAb SE144 is directed against the laminin γ2 chain [Vailly, 1994] and monoclonal antibody (mAb) K140 against the laminin β3 chain [Marinkovich, 1992]. PAb GOH3 is specific to integrin α6 [Sonnenberg, 1987]; mAb233 is directed against BP180 [Hieda et al., 1992], and mAb LH7:2 is specific to the collagen VII C-terminal domain (Sigma Immunochemical). PAb anti-laminin 1 (L9393, Sigma) and pAb anti-collagen VII (10411, Institut Pasteur, Paris, France) were also used. Secondary antibodies were FITC-conjugated goat anti-mouse Ig (Dako S.A., Trappes France), and goat anti-rat IgG (Cappel, ICN Biomedicals, Orsay,

France). The samples were processed as previously reported [Gache, 1996]. The tissue sections were examined using a Zeiss Axiophot microscope.

[0040] Reactivity to pAb SE144 specific to the laminin  $\gamma$ 2 chain, was absent in the affected foals (Figure 2a), while pAb SE85 and mAb K140 directed against the laminin  $\alpha$ 3 and  $\beta$ 3 chains, respectively, were faintly reactive (Figure 2c & e). Reactivity of collagen type VII (Figure 2g & h), integrin  $\alpha$ 6, collagen type XVII and laminin 1, was comparable to that observed in wild-type foals (not shown). These observations suggested that expression of laminin-5 was hampered in the affected animals and indicated that Lamc2, the gene encoding the laminin  $\gamma$ 2 chain, was involved in the etiology of the condition.

#### **Example 2 - Isolation of the horse laminin $\gamma$ 2 cDNA**

[0041] Biopsies were obtained from involved and non-involved areas of the skin and from tongue epithelia. Tissue samples were also obtained from a wild type non-related horse. The biopsies were snap frozen in liquid nitrogen and preserved at -70°C until processing. Total RNA was purified from the frozen skin biopsies using the RNable extraction kit (Eurobio, Les Ullis, France). Genomic DNA was purified from peripheral blood following standard methods [Sambrook, 1989]

[0042] Five  $\mu$ g of RNA (purified from frozen skin biopsies obtained from foal n° 1) was reverse transcribed in a volume of 25  $\mu$ l in the presence of 100 u of M-MLV reverse transcriptase (GIBCO-BRL, Life Technologies, Inc.). One  $\mu$ l of the reaction mixture was then used in PCR amplifications to obtain overlapping cDNA fragments spanning the open reading frame of the horse laminin  $\gamma$ 2 chain. Specifically, eight primer pairs were devised on the basis of the most conserved nucleotide sequence between the human (GenBank accession n° ZI5008 [Kallunki et al., 1992]) and mouse (GenBank n° NM 008485 [Sugiyama et al., 1995]) laminin  $\gamma$ 2 cDNAs (not shown). Direct sequencing of the different PCR amplification products resulted in the disclosure of 82% of the horse laminin  $\gamma$ 2 cDNA sequence. Primers specific to the horse  $\gamma$ 2 cDNA sequence were then designed to complete and verify the sequence of the full-length  $\gamma$ 2 cDNA (Table I). The PCR conditions were: 95°C for 5 minutes, followed by 35 cycles at 95°C for 40 seconds, annealing temperature (Table I) for 40 seconds, 72°C for 40 seconds, and a final elongation for 7 minutes at 72°C. The amplification products were purified using a QIAquick kit (Qiagen Madison, WI, USA), and subjected to automated nucleotide sequencing using an ABI Prism Model 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA).

[0043] To obtain the 5' end of the  $\gamma$ 2 cDNA, the sense oligonucleotide PCR 5'L corresponding to a 5' non-coding sequence of the human laminin  $\gamma$ 2 cDNA and the antisense primer PCR-5'R specific to

the horse  $\gamma 2$  cDNA sequence were used to amplify a 460-bp cDNA fragment (Table I). To obtain the 3' end of the  $\gamma 2$  cDNA, the antisense primer RT-PCR3' containing a polyT and tag sequence was used for RT-PCR amplification of total RNA. Subsequently two PCR reactions were performed; the first using primers PCR-3'L (position 3226 on the horse  $\gamma 2$  cDNA) and PCR3'R, which is composed of the tag sequence. The resulting 474-bp 3'-terminal cDNA fragment was used as template for a second PCR amplification using primer PCR-3A'L (position 3325 on the horse  $\gamma 2$  cDNA) and primer PCR-3'R. The PCR cycling conditions, purification and sequence analysis for the isolation of the 5' and 3' ends were done as previously mentioned. Analysis, alignment, and translation of the nucleotide sequence into the amino acid sequence were performed using the software, Lalign and Cluster W.

**Table II**

*Oligonucleotide primers used to amplify the horse laminin  $\gamma 2$  cDNA*

Primers	Position (a)	Position (b)	Nucleotide sequence (SEQ ID No.)	Annealing Temp. (°C)	Product size (bp)
1L	1		ATGCCTGCGCTCTGGCTCAG (3)	63	591
1R	592		TGTGGCAGCTGGCGGAATGC (4)		
2L	406		GACTCCAAGTGTGACTGTGA (5)	60	388
2R	794		TAGCTCACCTGTTGATTCCC (6)		
3L	742		CCTGTCTATTTGTAGCTCC (7)	57	670
3R	1412		CAGCTGAACCCATTGCGACA (8)		
4L	1003		GAGTATCGGAGGTTACTGCG (9)	59	756
4R	1759		GACACTCCACAGGCTCCGAG (10)		
5L	1477		CGCTGTGAGCTCTGTGCTGA (11)	65	422
5R	1899		CTCCAGGATCTGGAGCTGCT (12)		
6L	1705		GACAAGTGTGAGCTTGC (13)	60	395
6R	2100		TCATCATGAGGTCAATCCAGG (14)		
7L	1992		GAGAGAAAGCCCAGATTTCAC (15)	59	524
7R	2516		GCTTCCATGTGCGGTTGCGT (16)		
8L	2312		CAGCCAGTAACATGGAGCAA (17)	60	435
8R	2747		GTCTGTCTCCCATTCTTCC (18)		
9L	2658		GGATGAGTTCAAGCACGTG (19)	57	514
9R	3172		ACAGCTCTCCTTCACTTCT (20)		
10L	2955		CAAGACGAAGCAAGCAGAAG (21)	60	445
10R	3400		GGCTGTTGATCTGAGTCTTG (22)		
PCR-5'L	-197		GTGAGTCACACCCTGAAACA (23)	57	460
PCR-5'R	263		GAGTTACAATTGCAGGGTAAAC (24)		
RT-PCR 3'	-		GGCCATGCGTAGACTCTTAA(T) <sub>16</sub> (25)		
PCR-3'L	3226		GCAGAGGCCAAAGAGTTG (26)	57	474
PCR-3'R	-		GGCCATGCGTAGACTCTTAA (27)		
PCR-3A'L	3325		CCTGGCAGTGTGGATGAAGA (28)	57	375

(a) L, sense primer; R, antisense primer.

(b) The position number designates the 5' end of each primer in accordance with the horse laminin  $\gamma 2$  cDNA sequence (to be submitted to GenBank)

- [0044] The horse  $\gamma$ 2 cDNA was determined to comprise a 3570-bp full length open reading frame, a 197-bp 5' and a 222-bp 3' untranslated region (Figure 3). Computer assisted analysis of the nucleotide sequence revealed that the homology in the coding sequence between man and horse (89.1% identity) is higher than that between horse and mouse (83.3% identity) and that between man and mouse (83.8% identity). The 5' untranslated region (UTR) contains a GATAA box which is located -112 to -116 bp from the initiation ATG, and two AP-1 binding sites at position -129 to -135 and -170 to -176, respectively. Comparison of the 5'UTR of horse and human revealed that from position -62 to -197, which contains the regulatory motifs, the nucleotide sequence is identical, while from position -1 to -61 there is only 49% homology, with two base additions and seven base deletions. The 5' UTR of the mouse  $\gamma$ 2 cDNA is not available to include in this comparison [Salo et al., 1999].
- [0045] In the horse, the full-length  $\gamma$ 2 cDNA encodes a polypeptide of 1190 residues which is three amino acids shorter than in man (1193 aa), and two amino acids shorter than the mouse amino acid sequence (1192). Similar to the human and mouse  $\gamma$ 2 chain the ATG codon is followed by a signal peptide of 20 amino acids. In contrast, in the mouse only 18 hydrophobic amino acids corresponding to a putative signal peptide are found after the ATG codon. The position of the predicted signal peptide cleavage site (Ala 21) of the human  $\gamma$ 2 chain is conserved in the horse counterpart. The horse  $\gamma$ 2 polypeptide shows the domain structure similar to the mouse and human  $\gamma$ 2 chains where a N-terminal short arm, rich in EGF-like repeats, extends into a long rod-like C-terminal arm. The N-terminal short arm domain V (residue 28 to 196) consists of three and a half cysteine-rich EGF-like repeats; domain IV (residues 197-381) has a globular structure and contains a single cysteine; domain III (residues 382-608) comprises four and a half EGF-like repeats, and contains the proteolytic cleavage site YSGD [Gagnoux, 2001]. Domain I/II (residues 609-1190) constitutes the rod-like long arm of the polypeptide and is formed by heptad repeats typical of the  $\alpha$ -helical coiled-coil domains of the laminin chains. The horse  $\gamma$ 2 chain contains 67 cysteine residues and six putative N-glycosylation sites that are conserved in the man and the mouse amino acid sequence (Figure 4).
- [0046] Alignment of the amino acid sequences revealed that domains V, IV, III of the horse and human  $\gamma$ 2 chain are more than 90% identical, while homology between horse and mouse is 82.8%. Homology between domains I/II is significantly lower (81.3%) between horse and man, and between horse and mouse (76.6%) (Table II).

**Table II**

*Sequence identity (percentage) between the domains of the horse laminin  $\gamma 2$  chain and the human and mouse counterparts.*

	V	IV	III	I/II
Human $\gamma 2$	92.3	94	93	81.3
Murine $\gamma 2$	86.4	88.8	90.7	76.6

**Example 3 - Identification of genetic mutation that results in JEB in horses**

- [0047] Total mRNA extracted from skin biopsies of foal n° 1 was reverse transcribed in two separate reactions using in one, a universal oligo-dT and in the other, the nested primer 9R (Table I). Overlapping cDNA fragments spanning the full-length sequence of the  $\gamma 2$  cDNA were then amplified by PCR using the primer pairs listed in Table I. The PCR amplification products were purified and subjected to automated nucleotide sequencing. The  $\gamma 2$  cDNA sequence of the affected foal was compared with the wild-type nucleotide sequence using the software program Sequencher (Gene Codes Corp, Ann Arbor, Michigan). In the affected foal, the 756-bp cDNA fragment (nucleotides 1003 to 1759) obtained using primers 4L and 4R was found to contain a one-base pair insertion at position 1368 (mutation 1368insC; see Figure 5)
- [0048] Sequence chromatographic analysis following PCR amplification of the genomic DNA with the appropriate primers was used to readily distinguish homozygous (affected or wild-type) animals from heterozygous (carrier) animals. In the case of the homozygous affected horse, the chromatogram appears as a clear pattern of well-defined single peaks each of which correspond to the individual nucleotides of the sequence. The clear pattern of peaks is derived from both alleles of the nucleic acid since they are identical. At the point of the mutation there is an insertion of the nucleotide C in both alleles; this denotes the mutation as homozygous. A clear pattern also results in the case of a wild-type horse, in which both alleles are also identical. The resulting chromatogram in this case, however, represents a nucleic acid sequence which is not mutated by a "C" insertion. In the case of the heterozygous situation, the chromatogram shows two superposed sequences starting at the site of the insertion mutation, and corresponding to one allele having the inserted C (therefore, the sequence is shifted by one nucleotide), while the other is the wild type allele (see Figure 5).
- [0049] This mutation, designated 1368insC, causes a shift in the open reading frame of the  $\gamma 2$  messenger RNA and results in a downstream premature termination codon (TGA) at residue 476 which is in

the N-terminal portion of domain III, 41 residues downstream of the proteolytic cleavage site of the chain.

[0050] Identification of the mutation 1368insC at the genomic level was performed by PCR amplification of a 170-bp DNA fragment (nucleotides 1291-1461 of  $\gamma$ 2 cDNA sequence) using genomic DNA as a template and primers: (sense) 5'-TGTTACTCAGGGATGAGAA-3' (SEQ ID No: 29 – nucleotides 1291 to 1310 of the  $\gamma$ 2 cDNA sequence) and (antisense) 5'-CTGGGGGCAGTTATTGCAC-3' (SEQ ID No: 30 – the reverse compliment of the sequence from 1443 to 1461 of the  $\gamma$ 2 cDNA sequence), which correspond to the sequence within exon 10 of the human LAMC2 gene. PCR cycling conditions were: 5 minutes at 95°C, followed by 30 seconds at 95°C, 30 seconds at 56°C, 30 seconds at 72°C (35 cycles), and extension for 7 minutes at 72°C. After purification, the amplification product was submitted to automated nucleotide sequencing.

[0051] The presence of the homozygous mutation 1368insC in the other affected foals was confirmed. When the phenotypically healthy dam and sire of the affected foals were tested for the presence of this mutation, they were found to be heterozygous carriers. To assess the frequency of this mutation in the Belgian horse, samples for genomic DNA extraction were obtained from various breeding farms in the USA and Canada. Results demonstrated that 50% of the screened animals (n=132) were healthy carriers, which confirmed the Mendelian transmission of the mutation 1368insC (Figure 6).

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